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HUMAN PAPILLOMAVIRUS IS A NECESSARY CAUSE OF INVASIVE CERVICAL CANCER WORLDWIDE

JAN M. M. WALBOOMERS[1]*, MARCEL V. JACOBS[1], M. MICHELE MANOS[2], F. XAVIER BOSCH[3], J. ALAIN KUMMER[1], KEERTI V. SHAH[2], PETER J. F. SNIJDERS[1], JULIAN PETO[4], CHRIS J. L. M. MEIJER[1] AND NUBIA MUÑOZ[5]

[1]*Department of Pathology, University Hospital Vrije Universiteit, Amsterdam, The Netherlands*
[2]*Department of Molecular Microbiology and Immunology, Johns Hopkins University, Baltimore, MD, U.S.A.*
[3]*Institute Catala d'Oncologia, Barcelona, Spain*
[4]*Institute of Cancer Research, Belmont, Surrey, U.K.*
[5]*Unit of Field and Intervention Studies, IARC, Lyon, France*

SUMMARY

A recent report that 93 per cent of invasive cervical cancers worldwide contain human papillomavirus (HPV) may be an underestimate, due to sample inadequacy or integration events affecting the HPV L1 gene, which is the target of the polymerase chain reaction (PCR)-based test which was used. The formerly HPV-negative cases from this study have therefore been reanalysed for HPV serum antibodies and HPV DNA. Serology for HPV 16 VLPs, E6, and E7 antibodies was performed on 49 of the 66 cases which were HPV-negative and a sample of 48 of the 866 cases which were HPV-positive in the original study. Moreover, 55 of the 66 formerly HPV-negative biopsies were also reanalysed by a sandwich procedure in which the outer sections in a series of sections are used for histological review, while the inner sections are assayed by three different HPV PCR assays targeting different open reading frames (ORFs). No significant difference was found in serology for HPV 16 proteins between the cases that were originally HPV PCR-negative and -positive. Type-specific E7 PCR for 14 high-risk HPV types detected HPV DNA in 38 (69 per cent) of the 55 originally HPV-negative and amplifiable specimens. The HPV types detected were 16, 18, 31, 33, 39, 45, 52, and 58. Two (4 per cent) additional cases were only HPV DNA-positive by E1 and/or L1 consensus PCR. Histological analysis of the 55 specimens revealed that 21 were qualitatively inadequate. Only two of the 34 adequate samples were HPV-negative on all PCR tests, as against 13 of the 21 that were inadequate ($p<0{\cdot}001$). Combining the data from this and the previous study and excluding inadequate specimens, the worldwide HPV prevalence in cervical carcinomas is 99·7 per cent. The presence of HPV in virtually all cervical cancers implies the highest worldwide attributable fraction so far reported for a specific cause of any major human cancer. The extreme rarity of HPV-negative cancers reinforces the rationale for HPV testing in addition to, or even instead of, cervical cytology in routine cervical screening.

KEY WORDS—cervical carcinoma; HPV PCR; histology; HPV serology; epidemiology

INTRODUCTION

Cervical cancer is the second commonest cancer in women worldwide and infection with oncogenic human papillomavirus (HPV) types, most frequently HPV 16, is the most significant risk factor in its aetiology.[1] The International Biological Study on Cervical Cancer (IBSCC) study of invasive cervical cancers collected from 22 countries reported a worldwide HPV prevalence of 93 per cent, based on the MY09/11 polymerase chain reaction (PCR) assay which targets a 450 base pair (bp) fragment within the HPV L1 open reading frame (ORF).[2] Failure to detect HPV DNA in 7 per cent of these cervical carcinomas may have been due to either the absence of HPV DNA in the carcinoma cells, or a false-negative HPV result. Integration of HPV DNA in cervical carcinoma can entail disruption of PCR primer sequences or loss of the HPV L1 ORF.[3] In contrast, the E6 and E7 genes are almost invariably retained, as their expression is likely to be necessary for conversion to and maintenance of the malignant state.[4–8] A false negative can also be due to the absence of cancer cells from the sample analysed.

This study was undertaken to reassess the prevalence of HPV DNA in this worldwide series of cervical cancers. Since a marked association has been reported between cervical cancer and antibody responses against HPV-specific proteins,[9–13] serological analysis was first performed for capsid and E6 and E7 proteins of HPV 16, which accounts for about 50 per cent of HPV-positive cervical cancers worldwide.[2] To verify the presence of carcinoma cells in the samples used for PCR, formalin-fixed, paraffin-embedded biopsies of the HPV-negative cases were cut using a sandwich technique, in which the inner sections were used for PCR while the outer sections were used for histological review. The efficacy of amplification in paraffin-embedded tissue specimens is inversely related to the length of the fragment to be amplified.[14] The analysis was therefore restricted to samples which amplified at least 200 bp, as

*Correspondence to: Professor Dr. J. M. M. Walboomers, Department of Pathology, University Hospital Vrije Universiteit, De Boelelaan 1117, 1081 HV, Amsterdam, The Netherlands.
E-mail: JMM.Walboomers@azvu.nl

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determined by β-globin PCR. Adequate samples were assayed with 14 type-specific (TS) PCRs for high-risk HPV types targeting approximately 100 bp in the E7 ORF. In addition, CPI/II and GP5+/6+ consensus primer-mediated PCR assays were used, which also target small fragments. The PCR results were correlated with the histological findings.

MATERIALS AND METHODS

Clinical specimens

Sera from HPV-negative and a tissue sample from the HPV-positive cervical cancer patients were obtained from the IBSCC Study.[2] The HPV-positive cases were matched in frequency to the negative cases on age, histological type, clinical stage, and area of residence.

In the original analysis, 866 snap-frozen biopsies from patients with cervical cancer were HPV-positive and 66 were HPV-negative by MY09/11 PCR.[2] For the present study, formalin-fixed, paraffin-embedded tissue specimens were obtained from 58 of the 66 HPV-negative cases. Material from the remaining eight negative cases was not available.

A series of 5 μm sections were cut; the outer sections were used for histological analysis while the inner sections were used for PCR.[15] Depending on the size of the biopsy, five to ten sections were cut. For PCR analysis, tissue sections were pretreated as described previously.[16]

HPV serology

Antibodies to HPV 16 virus-like particles (VLPs) were detected by ELISA[17] and antibodies to HPV 16 E6 and E7 proteins were measured by a radioimmunoprecipitation assay (RIPA) with *in vitro* translated ^{35}S-labelled full-length E6 and E7 proteins.[11]

β-Globin PCR

β-Globin PCRs were performed[18] using four primer combinations spanning 100, 209, 326, and 509 bp to assess the quality of the DNA.

HPV E7 type-specific PCRs

For E7 PCRs, HPV type-specific oligonucleotides were selected on the basis of sequence information from the HPV sequence database[19] after alignment analysis using the Clustal program (PC/Gene, Release 6.7; Intelligenetics, Inc, Geneva Switzerland). HPV type-specific primers were chosen to amplify approximately 100 bp in the E7 ORF of HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68. Type-specific (TS) PCRs were performed under the conditions described by van den Brule *et al.*[20] HPV genotypes were identified by Southern blot hybridization using type-specific oligonucleotide probes. Reconstruction experiments using cloned HPV DNA serially diluted in human placental DNA revealed a sensitivity of between 10 and 100 HPV copies per sample for all primer sets (data not shown). Oligonucleotide sequences used as primers and probes are shown in Table I.

Consensus primer-mediated PCRs

Consensus PCR primers GP5+/6+[21] and CPI/II[22] were used for amplification. GP5+/6+ PCR was performed with one biotinylated primer (bioGP6+) to enable subsequent typing in an enzyme immunoassay.[23] CPI/II PCR was performed as described elsewhere.[22] Both consensus PCRs amplify a broad spectrum of HPV types at the subpicogram level. HPV positivity was assessed by Southern blot analysis of the PCR products with general probes of HPV-specific [α-^{32}P]dCTP-labelled DNA fragments derived from cloned DNA of HPV 6, 11, 16, 18, 31, and 33 as described previously.[21]

PCR products from GP5+/6+ PCR-positive specimens were typed using internal HPV type-specific oligonucleotide probes for the most common HPV types 16, 18, 31, and 33. The PCR products of CPI/II PCR-positive specimens were typed by stringent Southern blot analysis with [α-^{32}P]dCTP random-primed labelled full-length cloned HPV DNA of types 16, 18, 31, and 33 as described elsewhere.[24]

Negative controls for PCR analysis

Several precautions were taken to prevent false-positive results. Different steps such as sample preparation and amplification were performed in strictly separated rooms, and distilled water samples were included as negative PCR controls. To monitor sample-to-sample carry-over, HPV-negative liver tissue was cut after each specimen and subjected to all subsequent procedures including HPV PCR. None of these control samples was positive for any of the HPV assays.

Histological review

The first and last sections of each tissue were haematoxylin and eosin (H&E)-stained for histological analysis. The slides were examined by a pathologist without knowledge of the HPV status for the presence of neoplastic cells, extent of necrosis, extent of keratinization, and evidence of maltreatment (i.e. nuclear vacuolation suggestive of freeze/thaw damage). Final classification of equivocal specimens was made jointly with a second pathologist.

Statistical analysis

The Mann–Whitney *U*-test was used to compare OD and cpm values between HPV-positive and -negative cases. Histopathological characteristics were compared using Fisher's exact test. All significance levels are two-sided.

RESULTS

The 66 HPV-negative and 866 HPV-positive cases in the IBSCC study were similar in relation to established

Table I—Oligonucleotide sequences of HPV E7 type-specific PCRs

Primer	Sequence (5'–3')	Probe	Sequence (5'–3')
HPV16E7.667	gatgaaatagatggtccagc	PROHPV16E7	cggacagagcccattacaatattgtaacct
HPV16E7.774	gctttgtacgcacaaccgaagc		
HPV18E7.696	aagaaaacgatgaaatagatgga	PROHPV18E7	cccgacgagccgaaccacaacgtcacacaa
HPV18E7.799	ggcttcacacttacaacaca		
HPV31E7.811	gggctcatttggaatcgtgtg	PROHPV31E7	tacctgctggatcagccattgtagttacag
HPV31E7.890	aaccattgcatcccgtcccc		
HPV33E7.671	tgaggatgaaggcttggacc	PROHPV33E7	tgtgacaacaggttacaatgtagtaatcag
HPV33E7.761	tgacacataaacgaactgtg		
HPV35E7.674	ctattgacggtccagct	PROHPV35E7	caacaggacgttacaatattataattggag
HPV35E7.752	tacacacagacgtagtgtcg		
HPV39E7.601	ccaaagcccaccttgcagga	PROHPV39E7	tcctaattgctcgtgacatacaaggtcaac
HPV39E7.723	atggtcgggttcatctatttc		
HPV45E7.741	cccacgagccgaaccacag	PROHPV45E7	agctcaattctgccgtcacacttacaacat
HPV45E7.822	tctaaggtcctctgccgagc		
HPV51E7.718	tacgtgttacagaattgaag	PROHPV51E7	tcaagtgtagtacaactggcagtggaaagc
HPV51E7.841	aaccaggcttagttcgcccatt		
HPV52E7.691	gcagaacaagccacaagcaa	PROHPV52E7	atagccgtagtgtgctatcacaactgtgac
HPV52E7.776	tagagtacgaaggtccgtcg		
HPV56E7.784	ggtgcagttggacattcagag	PROHPV56E7	caaagaggacctgcgtgttgtacaacagct
HPV56E7.886	gttacttgatgcgcagagtg		
HPV58E7.98	cgaggatgaaataggcttgg	PROHPV58E7	tgttgttcaatgttacatcattaatcgaca
HPV58E7.761	acacaaacgaaccgtggtgc		
HPV59E7.646	ctccgagaatgaaaaagatgaa	PROHPV59E7	gtcgagcagatcgatcatcgtttcctacta
HPV59E7.749	gctgaagttgattattaca		
HPV66E7.641	aatgcaatgagcaattggacag	PROHPV66E7	aggatgaaatagaccatttgctggagcggc
HPV66E7.742	cttatgttgttcagcttgtc		
HPV68E7.4604	aacaacagcgtcacacaattca	PROHPV68E7	agtgtaacaacctactgcaactagtagtag
HPV68E7.4704	agttgtacgttccgcaggtt		

risk factors for cervical cancer (geographical origin, socio-economic status, sexual and reproductive lifestyle, exposure to cigarette smoke, hormonal treatments, and history of venereal disease).[2]

Serological analysis

Sera were obtained from 49 of the 66 HPV PCR-negative cases (group 1) and from a matched sample of 48 initially positive cases (group 2) from the previous study.[2] Reactivity to HPV 16 VLPs and E6 and E7 proteins was detected in 31 per cent (16/49), 35 per cent (17/49), and 29 per cent (14/49) of group 1 and in 42 per cent (20/48), 52 per cent (25/48), and 31 per cent (15/48) of group 2, respectively. Serum antibodies to at least one protein were detected in 56 and 67 per cent of groups 1 and 2, respectively. Positive serum samples tended to have lower antibody levels for VLPs and E6 in group 1 than in group 2 (Fig. 1), but there was no significant difference in percentage reactivity to VLPs ($p=0{\cdot}92$), E6 ($p=0{\cdot}30$) or E7 ($p=0{\cdot}76$) between the groups.

PCR analyses

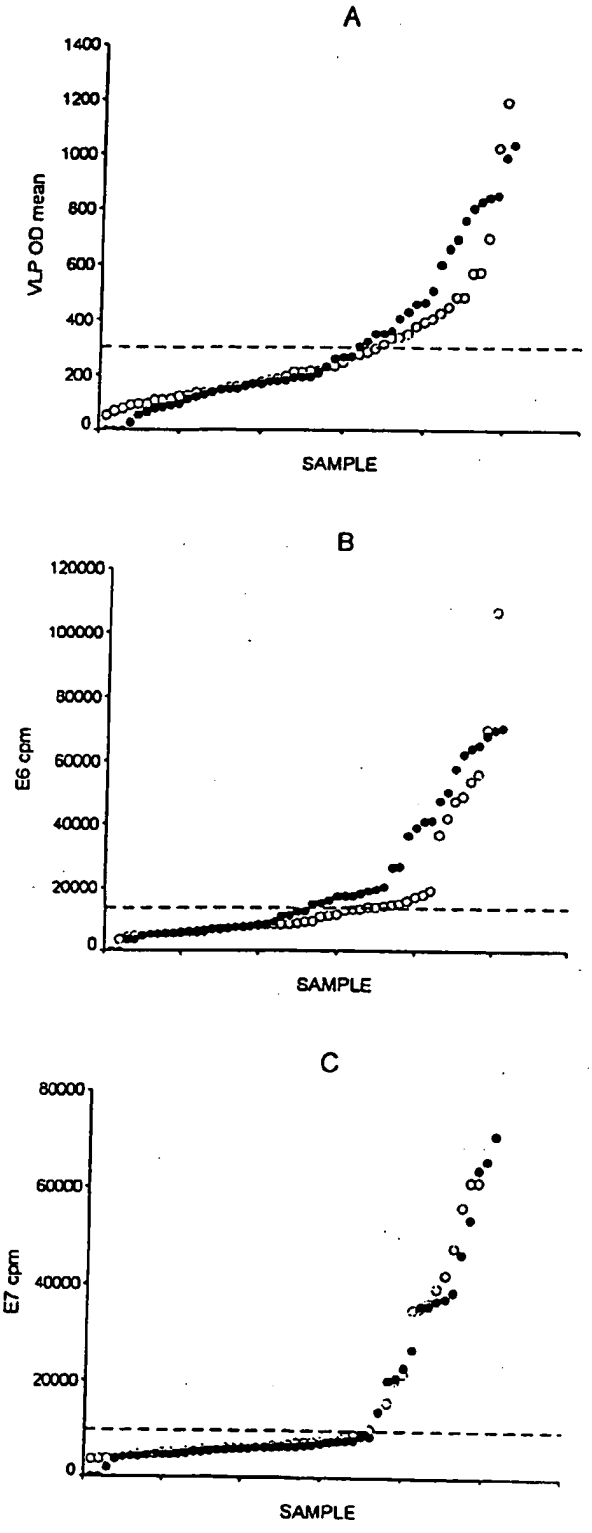
The integrity of the target DNA was determined by β-globin PCR assays amplifying different fragment lengths. Of the 58 cervical carcinoma specimens, 55 (95 per cent) were positive for amplification of β-globin fragments of 100 and 209 bp, and 43 (74 per cent) and 21 (36 per cent) cases were also positive for 326 and 509 bp fragments, respectively. The three cases which were negative with all four β-globin primer sets were excluded from the HPV PCR assays, all of which entailed amplification of sequences of 200 bp or less.

HPV E7 PCRs on the remaining 55 specimens were positive for one or more HPV types in 38 (69 per cent) cases. HPV 16 was detected in 15 cases; HPV 18 in nine cases; HPV 31 in four cases; HPV 45 in two cases; and HPV 33, 39, 52, and 58 in one case each. Multiple HPV infections were found in four cases: HPV 16/18 twice and HPV 18/45 and HPV 31/33 once each.

Using consensus PCRs, 14 samples were positive only with E1 primers, six only with L1 primers, and ten with both. Two cases were positive by consensus PCR but negative by E7 PCR. One of these was positive only for E1 and the other for both E1 and L1. These cases therefore contain an HPV type different from the 14 HPV types (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68) tested for by HPV E7 PCR. Tentatively, these types were referred to as HPV-X. The remaining 28 consensus PCR-positive samples were also positive by E7 PCR, and in every case in which typing of L1 and E1 PCR products indicated HPV 16, 18, 31 or 33, the same type was detected by E7 PCR.

Using HPV E7 type-specific and consensus L1 and E1 PCRs, the number of HPV-positive cases was thus 40/55

(73 per cent), while 15/55 (27 per cent) cases remained HPV-negative. The HPV detection and typing results are summarized in Table II.



Histological review of cervical carcinoma biopsies

The histological results and HPV status of the 55 biopsies are shown in Table III. Among the 40 HPV-positive cases, 31 were diagnosed as squamous cell carcinomas (SCC) and one as adenosquamous carcinoma. Six showed only dysplastic epithelium and the remaining two specimens were inadequate, one due to extensive necrosis (>75 per cent) and one due to maltreatment. Of the 32 HPV-positive histologically adequate cases, 24 (75 per cent) were positive on at least two different PCR assays. Two of the 15 HPV-negative cases were SCCs. The remaining 13 cases were characterized as having only dysplastic ($n=1$) or normal ($n=2$) epithelium, only stroma ($n=2$), extensive necrosis (>75 per cent; $n=2$), maltreatment ($n=3$) or extensive keratinization (>75 per cent; $n=3$). The histology of the HPV-negative group is shown in Fig. 2. The material was thus inadequate for eight (20 per cent) specimens in the HPV-positive group compared with 13 cases (87 per cent) in the HPV-negative group ($p<0{\cdot}001$). Figure 3 summarizes the PCR results in relation to adequacy of the tissue.

DISCUSSION

This study was designed to investigate the extent of true HPV-negative cervical cancer by retesting the cases originally classified as HPV-negative in a worldwide study of HPV prevalence in cervical carcinomas.[2] Samples from 58 of the 66 HPV-negative cases were available, and 55 were adequate for PCR analysis. The similarity of the antibody profiles of HPV-negative and -positive cases (Fig. 1) suggests that a significant proportion of the HPV-negative group were in fact HPV 16-associated. The epidemiological characteristics of the positive and negative cases were also similar. Our histological reassessment and PCR assays targeting different ORFs used cervical cancer samples prepared by the sandwich method, which addresses two potentially important causes of false-negative results: disruption of PCR target sequence due to viral integration, and inadequacy of the specimens.

The results of our consensus PCR assays targeting small fragments in the L1 (GP5+/6+ PCR) and E1 (CPI/II PCR) regions, which were positive in only about 50 per cent of the E7 PCR-positive cases (Fig. 3), suggest the presence of interruptions or deletions in the HPV DNA at the level of the L1 ORF. Our PCR assays achieve similar sensitivity for each HPV type, so these findings indicate that in the remaining E7-positive cases disruptions were present in both L1 and E1. Although integration assays are necessary ultimately to

Fig. 1—Antibody responses to HPV 16 VLP (A), E6 (B), and E7 (C) proteins of formerly HPV-negative (○) and HPV-positive (●) cases of cervical cancer. For VLP reactivity, mean extinctions (mean OD) and for E6/E7 reactivity, the counts per minute (cpm) are indicated. The cut-off levels for seropositivity (mean+3 × SD) for VLP (300), E6 (13 556), and E7 (9714) are indicated by dotted lines. Sera are arrayed in order of increasing reactivity. Each dot represents one serum specimen

Table II—Overall HPV prevalence and type distribution*

			HPV genotyping											
n	HPV −	HPV+	16	18	31	33	39	45	52	58	16/18	18/45	31/33	X†
55	15	40	15	9	4	1	1	2	1	1	2	1	1	2

*HPV prevalence was determined by HPV E7 type-specific PCR and consensus GP5+/6+ and CPI/II PCR assays. HPV genotyping was performed by HPV E7 type-specific PCRs.
†HPV-X: HPV types different from HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 as detected by GP5+/6+ and CPI/II PCR.

substantiate that integration is one of the underlying causes of false negativity, the suggestion that the disruptions are due to integration events was supported by further typing analysis. The prevalence of HPV 18 in the original analysis was more than three-fold lower than that of HPV 16 (13·7 per cent versus 49·9 per cent),[2] but amongst the E7-positive cases, HPV 18 was almost as prevalent as HPV 16 (22 per cent versus 31 per cent), suggesting that HPV 18 is more often disrupted in the L1 and/or E1 region. Previous studies[25,26] have shown that almost all HPV 18-positive cervical carcinomas contain only integrated HPV 18 DNA. In contrast, the proportion of HPV 16-positive cases that contain only integrated viral genomes is 60 per cent or less.[25–27] In a previous study, E7 ORF transcripts were detected in the neoplastic cells of all HPV 16 DNA-positive cervical carcinomas, including cases with very low HPV 16 copy number, provided that the RNA quality of the tissue was adequate.[15] This demonstration of continuous expression of the E7 ORF indicates an active role of E7 in the pathogenesis of cervical cancer. The samples in the present study were inadequate for RNA *in situ* hybridization (RISH), but the presence of E7 DNA in all 37 specimens in which DNA from a known HPV type was detected, including 23 without detectable L1 DNA and 16 without detectable E1 DNA (Fig. 3), supports the conclusion that E7 is required for maintenance as well as for initiation of the malignant state.[8]

Our PCR results thus indicate that many of the samples which were HPV-positive in this study were false-negative in the previous study due to integration events affecting MY09/11 L1 sequences, as suggested by Bosch *et al.*[2] It is most unlikely that the additional positives scored in this study are due to contamination. None of the negative controls included during sample preparation and PCR testing gave a positive result, and as the great majority were positive on two or more PCR assays, the results cannot be due to contamination with specific PCR products (Fig. 3). The strongest evidence, however, is our striking observation that a large majority (87 per cent) of HPV-negative cases, but only a minority (20 per cent) of HPV-positive cases (Table III: $p<0·001$), were histologically inadequate, which cannot be an artefact of contamination. This suggests that failure to detect HPV DNA in these cases in the original study, in which the presence of carcinoma was diagnosed in adjacent blocks, was due mainly to inadequate sampling. Histological inadequacy was defined in the present study by the presence of only normal or dysplastic epithelium or stroma, extensive necrosis, maltreatment, or extensive keratinization (Fig. 2). When analysis was restricted to samples which were histologically adequate, HPV was detected in 94 per cent (32/34) of the formerly HPV-negative carcinomas. The two remaining HPV-negative samples, which contained 50–75 per cent cancer cells and occasional necrosis, may be true HPV negatives, or might contain viral variants, sequence disruptions or very low HPV levels that escaped detection by the methods used.

Table III—Correlation of HPV status and histological characteristics

Histology	HPV+ (n=40) *N* (%)	HPV − (n=15) *N* (%)
Squamous cell carcinoma	31 (77·5)	2 (13·3)
Adenosquamous carcinoma	1 (2·5)	0 (0)
Only dysplastic or normal epithelium present	6 (15)*	3 (20)†
Extensive necrosis	1 (2·5)	2 (13·3)
Maltreatment	1 (2·5)	3 (20)
Only stroma present	0 (0)	2 (13·3)
Extensive keratinization	0 (0)	3 (20)

*All samples showed dysplastic epithelium.
†Two samples showed normal epithelium; one was dysplastic.

The great majority of cases in the IBSCC study were squamous carcinomas, but the conclusion that almost all cervical cancers contain HPV DNA is likely to be true irrespective of histology. Among cases which were adenocarcinomas or adenosquamous carcinomas, the prevalence of HPV by L1 consensus PCR was 95 per cent, the majority being HPV 18 or related types,[2] and the single adequate sample which was initially HPV-negative was shown in the present study to contain HPV E7 DNA (Table III). The IBSCC study included only 43 adenocarcinomas or adenosquamous carcinomas, but recent studies[28–30] using similar consensus PCR methods also detected HPV L1 DNA in 90 per cent or more of cervical cancers of these histologies, with HPV 18 being the most prevalent type. Our results suggest that HPV 18 is more often disrupted in the L1 region than other HPV types, which would imply that PCR assays targeting other ORFs are required to determine the true prevalence in adenocarcinomas.

The results of the IBSCC report together with the present study indicate that virtually all cervical cancers contain HPV DNA. In the original analysis, 981 samples were analysed by consensus PCR; 115 of these were

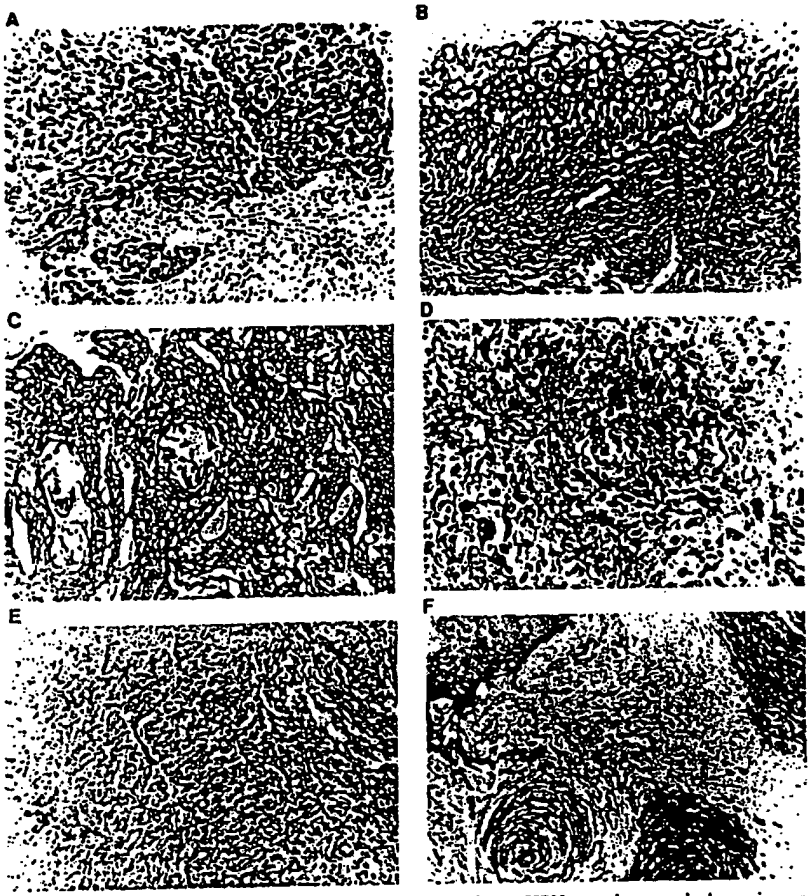


Fig. 2—Compilation of H&E-stained tissue sections from HPV-negative cervical carcinoma biopsies. (A) Squamous cell carcinoma; (B) only dysplastic/normal epithelium; (C) serious maltreatment, i.e. nuclear vacuolation; (D) extensive necrosis (>75 per cent); (E) only stroma; (F) extensive keratinization (>75 per cent)

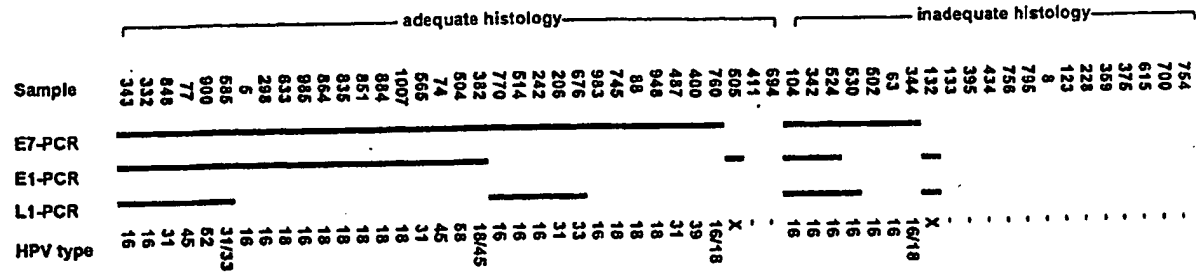


Fig. 3—HPV analysis of 55 cervical carcinomas using different PCR assays in relation to tissue adequacy. Bars indicate positivity for the respective PCR assay. HPV-X is HPV E7 type-specific PCR-negative but positive with one or both consensus PCR assays. E1 PCR: CPI/II PCR; L1 PCR: GP5+/6+ PCR

HPV-negative, of which 66 (57 per cent) were found on review to be adequate (containing tumour and PCR-sufficient).[2] Of these 66 samples, 58 were examined in the present study, and under our more stringent review (recutting multiple sections and accepting only cases with tumour in the outer sections and PCR-detectable β-globin DNA in the inner sections), 34 (59 per cent) were adequate, implying that only 34 per cent ($0{\cdot}57 \times 0{\cdot}59$) of the 115 originally negative specimens were adequate by this series of procedures. The proportion of the 866 initially HPV-positive specimens that would have been adequate by the same criteria is unlikely to be less than 80 per cent, the proportion that we observed among the 40 initially negative specimens which were found to be HPV-positive on retesting in the present study. On this assumption, the original study

included about 693 (80 per cent of 866) positive and 39 (34 per cent of 115) initially negative specimens which were adequate by our criteria. Our finding that only 6 per cent (2/34) of initially negative adequate specimens remained HPV-negative on retesting thus implies an overall HPV prevalence of 99·7 per cent among cervical cancers worldwide. Even if half of all women in the sampled populations had been infected with HPV at some time in their lives, this would indicate the highest worldwide attributable fraction ever identified for a specific cause of a major human cancer. The virtual absence of HPV-negative cancers implies that effective prophylactic vaccination might almost eliminate cervical cancer worldwide.[31] This is especially relevant in less developed countries, where screening may not be economically feasible. For developed countries, our results reinforce the rationale for HPV testing in combination with, or even instead of, cytology in population-based screening programmes.[32]

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REFERENCES

1. IARC. Monographs on the Evaluation of Carcinogenic Risks to Humans. Human Papillomaviruses, Vol. 64, Human Papillomaviruses. Lyon: International Agency for Research on Cancer, 1995.
2. Bosch FX, Manos MM, Muñoz N, *et al.* Prevalence of human papillomavirus in cervical cancer: a worldwide perspective. *J Natl Cancer Inst* 1995; 87: 796–802.
3. Walboomers JMM, Meijer CJLM. Do HPV-negative cervical carcinomas exist? *J Pathol* 1997; 181: 253–254.
4. Schwarz E, Freeze UK, Gissmann L, *et al.* Structure and transcription of human papillomavirus sequences in cervical carcinoma cells. *Nature* 1985; 314: 111–114.
5. Dürst M, Kleinheinz M, Hots M, Gissmann L. The physical state of human papillomavirus type 16 DNA in benign and malignant genital tumors. *J Gen Virol* 1985; 66: 1515–1522.
6. Choo K, Lee H, Pan C, *et al.* Sequence duplication and internal deletion in the integrated human papillomavirus type 16 genome cloned from a cervical carcinoma. *J Virol* 1987; 62: 1659–1666.
7. Wagatsuma M, Hashimoto K, Matsakura T. Analysis of integrated human papillomavirus type 16 DNA in cervical cancer: amplification of viral sequences together with cellular flanking sequences. *J Virol* 1990; 64: 813–821.
8. zur Hausen H. Molecular pathogenesis of cancer of the cervix and its causation by specific human papillomavirus types. In: zur Hausen H, ed. Human Pathogenic Papillomaviruses. Heidelberg: Springer-Verlag, 1994; 133–516.
9. Müller M, Viscidi R, Sun Y, *et al.* Antibodies to HPV-16 E6 and E7 proteins as markers for HPV-16-associated invasive cervical cancer. *Virology* 1992; 187: 508–514.
10. Viscidi R, Sun Y, Tsuzaki B, Bosch FX, Muñoz N, Shah K. Serologic response in human papillomavirus-associated invasive cervical cancer. *Int J Cancer* 1993; 55: 780–784.
11. Sun Y, Eluf-Neto J, Bosch FX, *et al.* Human papillomavirus (HPV)-related serologic markers of invasive cervical carcinoma in Brazil. *Cancer Epidemiol Biomarkers Prev* 1994; 3: 341–347.
12. Nonnenmacher B, Hubbert N, Kirnbauer R, *et al.* Serologic response to human papillomavirus type 16 (HPV-16) virus-like-particles in HPV-16 DNA-positive invasive cervical cancer and cervical intraepithelial neoplasia grade III patients and controls from Colombia and Spain. *J Infect Dis* 1995; 172: 19–24.
13. Hamšiková E, Novák J, Hofmannová V, *et al.* Presence of antibodies to seven human papillomavirus type 16-derived peptides in cervical cancer patients and healthy controls. *J Infect Dis* 1994; 170: 1424–1431.
14. Baay MFD, Quint WGV, Koudstaal J, *et al.* Comprehensive study of several general and type-specific primer pairs for detection of human papillomavirus DNA by PCR in paraffin-embedded cervical carcinomas. *J Clin Microbiol* 1996; 34: 745–747.
15. Walboomers JMM, Mullink H, Melchers WG, *et al.* Sensitivity of *in situ* detection with biotinylated probes of human papillomavirus type 16 DNA in frozen tissue sections of squamous cell carcinomas of the cervix. *Am J Pathol* 1988; 131: 901–905.
16. van den Brule AJC, Cromme FV, Snijders PJF, *et al.* Nonradioactive RNA *in situ* hybridization detection of HPV 16-E7 transcripts in squamous cell carcinomas of the uterine cervix using confocal laser scan microscopy. *Am J Pathol* 1995; 139: 1037–1045.
17. Viscidi RP, Kotloff KL, Clayman B, Russ K, Shapiro S, Shah KV. Prevalence of antibodies to HPV 16 virus-like particles in relation to cervical HPV infection among college women. *Clin Diagn Lab Immunol* 1997; 4: 122–126.
18. de Roda Husman AM, Snijders PJF, Stel HV, van den Brule AJC, Meijer CJLM, Walboomers JMM. Processing of long-stored archival cervical smears for human papillomavirus detection by the polymerase chain reaction. *Br J Cancer* 1995; 72: 412–417.
19. Myers G, Delius H, Icenogle J, *et al.* (eds). Human Papillomavirus Compendium: A Compilation and Analysis of Nucleic Acid and Amino Acid Sequences. Los Alamos, NM: Los Alamos National Laboratory, 1994, 1995 and 1996.
20. van den Brule AJC, Meijer CJLM, Bakels V, Kenemans P, Walboomers JMM. Rapid human papillomavirus detection in cervical scrapes by combined general primers mediated and type-specific polymerase chain reaction. *J Clin Microbiol* 1991; 28: 2739–2743.
21. de Roda Husman AM, Walboomers JMM, van den Brule AJC, Meijer CJLM, Snijders PJF. The use of general primers GP5 and GP6 elongated at

their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by polymerase chain reaction. *J Gen Virol* 1995; 76: 1057–1062.

22. Tieben LM, ter Schegget J, Minnaar RP, *et al.* Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. *J Virol Methods* 1993; 42: 265–280.
23. Jacobs MV, Snijders PJF, van den Brule AJC, Helmerhorst ThJM, Meijer CJLM, Walboomers JMM. A general primer GP5+/6+ mediated PCR–enzyme immunoassay method for rapid detection of 14 high risk and 6 low risk human papillomavirus genotypes in cervical scrapings. *J Clin Microbiol* 1997; 35: 791–795.
24. de Roda Husman AM, Walboomers JMM, Meijer CJLM, *et al.* Analysis of cytomorphologically abnormal cervical scrapes for the presence of 27 mucosotropic human papillomavirus genotypes using polymerase chain reaction. *Int J Cancer* 1994; 56: 82–86.
25. Cullen AP, Reid R, Campion M, Lörincz AT. Analysis of the physical state of different human papillomavirus DNAs in intraepithelial and invasive cervical neoplasm. *J Virol* 1991; 65: 606–612.
26. Berumen J, Casa L, Segura E, *et al.* Genome amplification of human papillomavirus types 16 and 18 in cervical carcinomas is related to the retention of E1/E2 genes. *Int J Cancer* 1994; 56: 640–645.
27. Matsukura T, Koi S, Sugase M. Both episomal and integrated forms of human papillomavirus type 16 are involved in invasive cervical cancers. *Virology* 1989; 172: 63–72.
28. Chichareon S, Herrero R, Muñoz N, *et al.* Risk factors for cervical cancer in Thailand: a case-control study. *J Natl Cancer Inst* 1998; 90: 43–49.
29. Ngelangel C, Muñoz N, Bosch FX, *et al.* The causes of cervical cancer in the Philippines: a case-control study. *J Natl Cancer Inst* 1998; 90: 50–57.
30. Chaouki N, Bosch FX, Muñoz N, *et al.* The viral origin of cervical cancer in Rabat, Morocco. *Int J Cancer* 1998; 75: 546–555.
31. McNeil C. HPV Vaccines for cervical cancer move toward clinic, encounter social issues. *J Natl Cancer Inst* 1997; 89: 1664–1666.
32. Meijer CJLM, Rozendaal L, van der Linden JC, Helmerhorst ThJM, Voorhorst FJ, Walboomers JMM. Human papillomavirus testing for primary cervical cancer screening. In: Franco E, Monsenego J, eds. New Developments in Cervical Cancer Screening and Prevention. Oxford: Blackwell Science, 1997; 338–347.